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Isolation and characterization of *Helicobacter pylori* recovered from gastric biopsies under anaerobic conditions

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Abstract

Background and Aim—*H. pylori* can survive long incubation periods under anaerobic conditions, and should be possible to isolate under anaerobic conditions. Our aim was to isolate *H. pylori* in anaerobic conditions, from gastric biopsies of *H. pylori* infected patients.

Methods—We enrolled 27 patients with bleeding (erosive) gastritis (mean age 36.3 years, 55.6% male) from Hanoi, Vietnam. *H. pylori* status was confirmed by qPCR.

Results—*H. pylori* were recovered under anaerobic and micro-aerobic conditions from gastric biopsies in 16 patients. Anaerobic conditions yielded significantly higher *H. pylori* recovery rates than micro-aerobic conditions (81.3% vs. 31.3%, p=0.01). *H. pylori* isolates were characterized by PCR for specific virulence markers and the genotypes were similar to those previously described in this region of the world.

Conclusions—*H. pylori* can be isolated under anaerobic conditions. These findings may provide new insight into the physiology of this human pathogen and help to identify the route of *H. pylori* transmission.

Keywords

bacterial culture; anaerobic conditions; *H. pylori* isolation

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INTRODUCTION

Helicobacter pylori is the leading gastric pathogen that colonizes the human stomach and causes a spectrum of diseases such as chronic active gastritis, gastric ulceration, MALT lymphoma and gastric cancer among others [1–5]. The role of the bacteria as a human pathogen has been confirmed in many parts of the world [6, 7].

Helicobacter pylori is recognized as an inhabitant of the human gastric mucosa. The organism has been regarded as microaerophilic, as its optimal growth occurs in the presence of 5–15% oxygen [8]. In general, primary cultures of *H. pylori* have less oxygen tolerance with a growth maximum at 3–7% O_2 [3, 9]. Most studies with standardized atmospheres for culturing *H. pylori* have used 2–5% O_2, 5–10% (optimal closer to 10%) CO_2 and 0–10% H_2 [10, 11]. Subcultures of *H. pylori* can rapidly be adapted to grow aerobically in a standard CO_2 mixture (18% O_2, 10% CO_2) in an incubator [12]. In addition*, H. pylori* appear to survive in other gas environments such as 10% CO_2 alone or variations in oxygen levels [8, 13].

There has been no consensus about the specific oxygen and carbon dioxide needs of *Helicobacter pylori.* Some investigators do not consider the bacteria a true micro-aerobic, as *H. pylori* is also a capnophile that grows equally well *in vitro* under micro-aerobic or aerobic conditions at high bacterial concentrations, and behaves like an oxygen-sensitive microaerophilic at low cell densities [14]. In 1999, Yamaguchi reported that *H. pylori* strain IK1029 growing under microaerophilic condition was able to survive long incubation periods under anaerobic conditions [15]. Furthermore, *H. pylori* has also been cultured from the feces [16, 17] that form in the anaerobic large intestine. These reports suggest that *H. pylori* may grow under anaerobic conditions.

To determine whether *H. pylori* can grow in both an anaerobic and micro-aerobic environment, we incubated biopsy homogenates from *H. pylori* infected and not infected patients under each condition. We report here that *H. pylori* can be isolated as a primary culture under anaerobic conditions as well as under micro-aerobic conditions. We also report that the genotypes of the isolated *H. pylori*, characterized by traditional PCR, are consistent with the genotypes previously described in this region of the world, independent of culture conditions.

METHODS

1.1. Biopsy specimens

Patients enrolled in Buu Dien Hospital were interviewed and signed informed consent forms before undergoing endoscopic examination to monitor stomach lesions. Patients' consent forms were approved by a local committee at the Buu Dien Hospital. Gastric biopsies from 27 patients were taken from antrum (AB) and corpus (CB) and flash-frozen immediately in liquid nitrogen for further study without cryopreservative. In addition, gastric juice was collected from all patients and pH was measured using pH paper.

1.2. Microbiological methods

1.2.1, Medium and processing of gastric biopsy samples—Non-selective medium commercial 5% Sheep Blood Agar was used for culturing *H. pylori*. Gastric biopsies were processed as previously described [18]. The delay between the removal of the specimens from the freezer and the inoculation onto culture media did not exceed 2–4 h. Gastric biopsies were homogenized in 300 μl of sterile 1× PBS and then 30 μl of the suspension was spread on the surface of non-selective blood media. The rest of the biopsy homogenates were used for total genomic DNA purification.

1.2,2, Culture condition—Plates were incubated at 37°C under two different atmospheric conditions: anaerobic and microaerophilic, using the BD Gas Pak™ EZ gas generating container systems (BD Company, Sparks MD) (anaerobic) or the BD Campy container system (microaerophilic), respectively. *H. pylori* growth was monitored from days 3–7. Bacterial morphology was examined by Gram staining at a magnification of ×1000. Bacterial isolates recovered from anaerobic and microaerophilic conditions were confirmed as *H. pylori* on the basis of positive urease reaction, typical colony morphology (small, round colonies) and traditional PCR analysis for several bacterial genes including *cagA, vacA, hspA, 23SrRNA* and the intergenic region between *jhp0153* and *jhp0152*. Furthermore, all *H. pylori* isolated recovered from anaerobic and microaerophilic conditions were plated again under the same atmospheric conditions to confirm their ability to grow in anaerobic and microaerophilic conditions.

1.3. Molecular methods

1.3.1, DNA purification—Total genomic DNA was purified from the biopsy homogenates using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia CA). Bacterial DNA was purified from strains isolated under anaerobic and microaerobic conditions using the MBI GeneJet Genomic DNA purification Kit (Fermentas, Vilnius, Lithuania). Concentration of extracted purified DNA was determined using the NanoDrop 1000 (Thermo Scientific, Hanover Park IL).

1.3.2, PCR amplification—PCR amplification occurred with 1–2 μl (100–200ng) of purified DNA from biopsies or bacteria. Each PCR reaction contained 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 200 μM each dNTP, 25pmol each primer, and 2.5 Unit of Taq polymerase. Temperature and step durations are included in Table 1. The reactions were conducted for 30–35 cycles using the Hybaid Thermocycler MBS 0.5G (Thermo Electron Corporation, Marietta OH). The PCR products were confirmed for size and purity on 1.5%–2% agarose gel run with 1× TAE buffer. The primers used in the analysis were shown in the Table 1.

cagA: The 3'end of the *cagA* gene was amplified with two primers, one of them degenerate [19]. The carboxyl terminal portion of CagA protein has multiple phosphorylation sites, and there is a clear difference in the number and type of phosphorylation sites between *H. pylori* strains isolated from Eastern and Western countries [20]. PCR conditions: 5-min hot start, 25 to 30 cycles as follows: 94°C for 30 s, 56°C for 30 s, 72° C for 1 min. For those strains that were *cagA* negative, we confirmed the lack of the pathogenicity island by empty-site PCR.

1.3.3, qPCR Methodology

A set of primer, and a TaqMan MGB probe specific for *H. pylori* were developed based on the *H. pylori 16S rRNA* gene. Forward primer: 5’-AGAGACTAAGCYCTCCAACAAC; Reverse primer: 5’-AATACTCATTGCGAAGGCGA; and Probe: 5’-TACGGGAGGCAGCAGT. Standards for total bacteria and *H. pylori* were prepared from the 16S rRNA, which were amplified from *H. pylori* strain 26695 using the bacterial 8F/1510R primer [21]. PCR products were cloned into the pGEMT easy vector (Promega, Madison, WI), and confirmed by sequencing. The qPCR-mix, with a total volume of 20 μl per well, consisted of 5 μM of forward and reverse primers, 2 μM MGB probe, 10μl Light Cycler 480 Probes Master (Roche), and 1 μl extracted DNA. The thermal cycle program consisted of 45 cycles of amplification at 95°C for 10s, 54°C for 30s, and then 72°C for 20s, with an initial cycle of 95°C for 10 min. The assays were performed using the Roche LightCycler 480 II PCR system, run in duplicate, and results analyzed using the LightCycler480 II program (Roche). We used 10-fold dilutions of the cloned *16S rRNA* gene from *H. pylori* strain 26695 to build standard curves to estimate the quantity of *H. pylori.* We also estimated the quantity of total bacteria from DNA purified from gastric biopsies using a previously described method [21].

1.3.4, PCR amplification of virulence markers

Empty site—The test for empty-site, related to the presence of the PAI Island and *cagA* gene in *H. pylori* genome, was performed in *cagA* negative strains [22]. Determination of *vacA* genotypes. The *vacA* genotype (s1/s2 or m1/m2) of each strain was evaluated according to the method of [23]. The primers used in this study for *vacA* m1/m2 genotyping were designed in our laboratory for this study. *HspA* (heat shock protein gene). The fragment of *H. pylori hspA* gene was amplified as previously reported [24].

Intergenic region between *jhp0153* and *jhp0152*—A PCR reaction amplified an intergenic region from the *H. pylori* genome as previously reported [25]. Based on the product size amplified, we could identify *H. pylori* strains of African origin by the distinctive 180bp insert that they carry in this intergenic region [25].

23S rRNA—Full length of *23S rRNA* gene was amplified with primers as previously described [26].

1.4. *H. pylori* infection confirmation

The *H. pylori* status of the patients was confirmed based on the results of qPCR and culture-based methods. The patient's *H. pylori* status was considered positive if qPCR results were positive in the purified DNA extracted from the gastric biopsy and the *H. pylori*/total bacteria ratio assessed by qPCR was >30% based on our validation experiment that included 25 well known positive *H. pylori* subjects and 20 well known *H. pylori* negative subjects (data not shown). Patients were considered *H. pylori* negative, if PCR results performed on purified gastric biopsy DNA were negative and the ratio of *H. pylori* to total bacteria by qPCR was <30%.

1.5 Non-*H. pylori* organisms identification

Single colonies of non-*Helicobacter pylori* appearing on the same blood-agar plates with *H. pylori* were selected from the agar surface and purified by sub-culturing several times using TSA agar plates with 5% sheep blood, incubated under anaerobic and aerobic conditions, and subsequently incubated at 37°C for 48 hours prior to DNA extraction. We next performed PCR of the 16s rRNA gene using bacteria universal primers previously described [27]. Next we performed purification of the PCR product, and 16S rRNA sequencing using Sanger method (Macrogen, NYC, US) [22, 26]. DNA was extracted using genomic DNA purification kit (Fermentas K0722).

RESULTS AND DISCUSION

2.1 Demographic and clinical characteristics of patients

Over a study period of 5 months, from December 2010 to April 2011, 54 gastric mucosal biopsy specimens were obtained from 27 patients with gastric bleeding (15 male, 12 female). The mean age of the group was 36.3 years, with a range of 25 to 57 years of age. Gastric juice pH was found as expected to be acidic for all patients with a mean value of 2 and a range between 1 and 4, except for one patient with a gastric juice pH of 8.0.

2.2 H. pylori infection of the patients confirmed by qPCR

The *H. pylori* infection of the patients was determined by qPCR using genomic DNA extracted from the gastric biopsies. It was confirmed by calculating the proportion of *H. pylori* present in the biopsy to the total number of bacteria estimated by qPCR in the same sample. We defined as a threshold of positivity that the proportion of *H. pylori* was greater than or equal to 30%. The *H. pylori* infection status of patients is presented in Table 2. Twenty-one (77.8%) patients were determined as *H. pylori* positive and 6 patients as *H. pylori* negative by quantitative PCR. Nearly all subjects with at least one biopsy sample positive by culture were also positive by qPCR with the exception of one patient (6.25%). In addition, of the 23 biopsies with culture positive, 4 (17.4%) were qPCR negative. These discrepancies are due to the patchy distribution of the infection. Regardless of the criteria used to define *H. pylori* status, biopsies obtained from the antrum were more informative of the *H. pylori* status than biopsies obtained from the corpus. However, the difference in reliable *H. pylori* status determined from these body sites was not significant. Our results confirmed the relevance of molecular methodologies for the diagnostic of *H. pylori* [28].

2.3 Primary isolation of H. pylori under anaerobic and microaerophilic conditions

Table 3 shows the recovery rates of *H. pylori* strains from biopsies obtained from 21 *H. pylori* infected-patients with gastric bleeding under microaerophilic and anaerobic conditions. The results indicate that an anaerobic environment coupled with blood agar provides growth conditions that yield the highest *H. pylori* recovery rate when compared with microaerophilic conditions. All the micro-aerobic positive samples were also positive in anaerobic culture conditions. The recovery of *H. pylori* under anaerobic was higher than under microaerophilic conditions (81.3% vs.31.3%, p value <0.01 Fisher Exact Test). All the isolated colonies under anaerobic conditions were able to grow under the same anaerobic conditions outside of the primary isolation using the same methodology for generating anaerobic conditions.

Non-*H. pylori* bacteria strains appeared to occur in the same plates with *H. pylori* under anaerobic conditions. They were recovered and identified by *16S rRNA* analysis as facultative anaerobic bacteria *Streptococcus* spp. (*S. australis, S. infantis* and *Bacillus spp.* (*B. licheniformis, B. subtilis, B. cereus, B. megaterium*). The remaining non-*H. pylori* bacterial isolates were identified as *Ochrobactrum intermedium, Rothia mucilaginosa* and *R. dentocariosa.*

2.4 Genotyping H. pylori isolates

Of the 23 isolated H. pylori strains, DNA was purified from 20 (87%) *H. pylori* isolates obtained from 14 of the 16 patients with a positive *H. pylori* status. Each strain was genotyped (Table 4). In six out of the 14 patients (42.9%), *H. pylori* strains were recovered from both sites of the stomach (antrum and corpus). For the remaining 8 patients, *H. pylori* strains were recovered from only one site. The presence of CagA positive strains is nearly universal among patients from East Asian countries colonized with *H. pylori*, according to Yamaoka *et al* and Truong *et al* [29, 30], and as expected, 80.0% (16 of 20) of *H. pylori* isolates from this Vietnamese population were *cagA* positive. We sequenced the 3’ variable region and confirmed that all the strains possess the Eastern D motif at the carboxyl terminal region of CagA, as has been previously reported from East Asian countries [20, 31]. Interestingly, isolates obtained from two patients (8 and 21) lacked the *cagA* gene according to PCR analysis. However, these same isolates showed evidence of carrying the Cag pathogenicity island. We did not test further to determine if those strains harbored a partial Type Four Secretion System (T4SS).

All 20 *H. pylori* strains included the16 strains that tested positive for the *cagA* gene were *vacA* positive. Of these strains, twelve (75%) showed the s1/m2 genotype and 4 showed the s1/m1 genotype. Some of the *H. pylori* strains showed multiple alleles, despite single colonies being picked. DNA was purified from only one of these strains for testing by PCR, thus, we cannot rule out the possibility of mixed infection in those patients with multiples *vacA* alleles. A possible mixed infection may also explain the case of patient 17 with *cagA* positivity but empty-site positivity. All *H. pylori* isolates in the study were confirmed to bear the intergenic region between *jhp0153* and *jhp0152* in their genome. However, none of the *H. pylori* isolates carried the 180bp insert that has been associated with strains of African origin [24]. The intergenic region we observed has been reported mostly in *H. pylori* strains isolated from East Asian countries such as Japan and Korea [24].

Humans are the major reservoir of *H. pylori,* where the bacteria appears to occur along certain areas of the gastrointestinal tube, including dental plaque, the esophagus, stomach, and gastric metaplasia into small intestine and large intestine [7, 17, 32–35].

In this study we provide evidence that *H. pylori* not only survives under anaerobic conditions, as Yamaguchi described [15], but that it can grow on non-selective blood agar under the anaerobic conditions generated by the BD Gas Pak. Furthermore, the genotypes of the *H. pylori* recovered are consistent with the genotypes previously described in Vietnam [36–38].

H. pylori acquisition occurs in early infancy via oral-oral or oral-fecal routes [39–41]. In addition, *H. pylori* have been isolated by culture method from human feces under micro-aerobic conditions, and now this study has confirmed that *H. pylori* can also grow in anaerobic conditions. *In vivo*, the bacteria might colonize the anaerobic large intestine microbiota or be washed from the upper digestive tract (stomach) and distributed along the human digestive tube, where *H. pylori* continues to grow approximately 3–4 hours within the large anaerobic intestine [42, 43] before being excreted in feces into the external environment. The fecal-oral transmission via water - or waterborne transmission - might be probable in the spread of infection in human communities, especially in crowded ones with inadequate sanitation.

The findings of this study may provide new insight into the physiology of this fastidious human pathogen and inform future epidemiological studies covering the route of *Helicobacter pylori's* transmission.

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Highlights

- We were able to growth *Helicobacter pylori* under anaerobic conditions.
- The use of anaerobic pack yield better recovery of *H. pylori* than Campy pack.
- No difference in genotype or phenotype was observed between the bacteria growth in either condition.

Table 1

Primers used for PCR and qPCR

Gene target	**Primers**	**Reference**	**Temperature and step**
cagA	5’-GTTAARAATRGTGTRAAYGG -3’ 5’-TTTAGCTTCTGATACCGC-3’	19	94°C 30s, 56°C 30s, 72°C 1min.
Empty-site	5’-CTCTTTTTGTGCCTTTGATTGAA-3’ 5’-CCAAATACATTTTGGCTAAATAAAC-3’	21	94°C 30s, 54°C 45s, 72°C 1min.
hspA	5’GCTATCTGAAAATTTGATTTCTTTTGC-3’ 5’-TGCGCTATAGTTGTGTCGC-3’	23	94°C 30s, 52°C 30s, 72°C 2min.
Intergenic region	5'-GTGGCGCGTTTCTTGCAATACC-3' 5'-AACTCGCTCAAAAACTCGGC-3'	24	94°C 30s, 57°C 30s, 72°C 1min.
23S rRNA	5’-CTCCATAAGAGCCAAAGCCC-3’ 5’-CCACAGCGATGTGGTCTC-3’	25	94°C 60s, 60°C 45s, 72°C 1min.
VacA status			
vacA s-region	5’-TGAGTTGTTTGATATTGAC-3’ 5’-CAATCTGTCCAATCAAGCGAG-3’	22	94°C 30s, 50°C 45s, 72°C 45s.
vacA m-region	5’-CTGCTTGAATGCGCCAAAC-3’ 5’-ATGGAAATACAACAAACACAC-3’	This study	94°C 30s, 52°C 30s, 72°C 2min.
qPCR			
Hp 16S rRNA	5’ –AGAGACTAAGCYCTCCAACAAC-3 5’ –AATACTCATTGCGAAGGCGA-3’	This study	
	HP probe 5’ -CATTACTGACGCTGATTGYGC	This study	
Eubacteria 16s rRNA	5’ –CAGCAGCCGCGGTRATA-3 5’ –CGYCAATTGBGBAADATTCC-3’	26	
	Eubacteria probe 5’ –TACGGGAGGCAGCAGT-3’	26	

Table 2

H. pylori infection of the patients confirmed by qPCR and culture method

Biopsy location	**qPCR positive**		**Culture positive**	
	n	**%**	**n**	**%**
Antrum and Corpus	8	29.6	7	25.9
Antrum only	10	37.0	6	22.2
Corpus only	3	11.1	3	11.1
Either	21	77.8	16	59.3

Table 3

Recovery rates of *H. pylori* strains in 16 culture positive patients under microaerophilic and anaerobic conditions

Biopsy location	**Microaerobic environment**		**Anaerobic environment**	
	n	**%**	**n**	**%**
Antrum and corpus	0	0	7	43.8
Antrum only	3	18.8	4	25.0
Corpus only	2	12.5	2	12.5
Either	5	31.3*	13	81.3*

Chi square Fisher exact test p values < 0.01

Table 4

Genetic characterization of *H.pylori* strains isolated from gastritis patients

Patient	Biopsy location	Intergenic region*	*cagA*	Empty site**	*hspA*	*vacA* s/m	Culture conditions
1	A/C	−	+		+	s1/m2	Anaerobic
3	A/C	−	+		+	s1/m1/m2	Micro/Ana
4	A	−	+		+	s1/m1	Anaerobic
5	A/C	−	+		+	m1/m2	Anaerobic
7	A/C	−	+		+	s1/m2	Anaerobic
8	C	−	−	−	+	s1/m2	Micro/Ana
17	A	−	−	+	+	s1/m2	Anaerobic
19	C	−	+		+	s1/m1/m2	Micro/Ana
21	A	−	−	−	+	s1/m2	Anaerobic
23	A	−	+		+	s1/m2	Micro/Ana
24	A/C	−	+		+	s1/m2	Micro/Ana
25	A	−	−	+	+	s1/m2	Anaerobic
26	A/C	−	+		+	s1/m1	Anaerobic
27	A	−	+		+	s1/m1	Anaerobic

A= antrum
C= corpus
s= signal sequence
m=middle region
Micro=micro-aerobic conditions
Ana=anaerobic conditions

* Negative for the 180bp insert

** Empty-site PCR was performed only on *cagA* negative strains in order to determine presence or absence of the *cag* pathogenicity island. The presence of a band in the empty site PCR represents the lack of Pathogenicity Island.